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Absolute $CD4^+$ Cell Count Using a Plastic Microchip and a Microscopic Cell Counter

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We have designed and evaluated the performance of a simple, rapid, and affordable method for counting $CD4^+$ T-cells with the use of plastic microchips. This new system is an adaptation of a "no-lyse, no-wash," volumetric single platform assay, and absolute $CD4^+$ counts are determined with the use of a microscopic scanning cell counter. To assess the $CD4^+$ count test precision and linearity of the system, measured $CD4^+$ counts were compared with two other reference assays (single and dual platform flow cytometry) with the use of 123 clinical samples including samples obtained from 35 HIV-infected patients, and artificially diluted samples. A correlation between the results from the use of the new method and from the use of the two other reference assays was $r = 0.98$ for the clinical samples. A dilution test of the new method demonstrated a linearity of $r \geq 0.99$, with coefficients of variation $\leq 7.6\%$ for all concentration levels. Our findings suggest that the new $CD4^+$ counting device can be potentially be applied for other diagnostic procedures that measure quantities of characteristic antigens or other materials on cells. © 2009 Clinical Cytometry Society

Key terms: antigens; CD4; CD4 lymphocyte count; flow cytometry; microchip; absolute CD4 count; microscopic cell counter

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The incidence of HIV infection continues to increase rapidly in the developing world, making it one of the most destructive epidemics in history (1). Currently, more than 35 million people in developing countries are living with HIV infection. Despite considerable prevention efforts in recent years promoted by the World Health Organization (WHO), the prevalence of HIV continues to increase in developing countries (1,2).

Determination of the count of $CD4^+$ T cells in peripheral blood is essential for staging HIV-infected patients, to decide the need for antiretroviral medications and to

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monitor the course of infection (3–5). During therapy, the presence of adequate $CD4^+$ T lymphocyte counts is indicative of the success of the therapy (3). The precise determination of $CD4^+$ T cell counts by flow cytometry using a technique for fluorescence-based measurement of immuno-labeled cells has been designated as the 'gold standard' for CD4 counting by the WHO (3). However, CD4 counting by flow cytometry is mostly unavailable in developing countries because of the complexity of the method and an associated high cost, whereas manual methods are extremely labor intensive, have a low throughput and are less accurate than the use of traditional flow cytometry methods (5–8). Therefore, efforts have been made to develop alternative and affordable $CD4^+$ T cell counting methods for resource-poor settings (9–14). These recently introduced $CD4^+$ T cell counting methods, however, also have several limitations that include expensive instrumentation, high reagent costs, and, in most cases, technical difficulties.

We have developed a $CD4^+$ T lymphocyte-counting device using a microchip and a previously described microscopic cell counter (15). This system adopts a "no-lyse, no-wash" procedure and counts $CD4^+$ cells in a given volume using a single CD4 antibody. The fluorescence signal emitted from the $CD4^+$ cells is displayed as a captured optical image. We have compared the use of the new method for $CD4^+$ T lymphocyte counting to the use of single platform and dual platform flow cytometry methods and have measured the accuracy and precision of the new method.

MATERIALS AND METHODS

Plastic Microchips

Microfluidic chip design and computer simulation. We designed the microfluidic chip to have even distribution of the cell density through the channel and to generate natural capillary flow for sample loading. To validate the design concept, we performed Computational Fluid Dynamics (CFD) simulations with the use of a multiple-physics software package based on the Finite-Volume Method (CFD-ACE (U) version 2003, CFD Research Corporation, Huntsville, AL). The Volume-Of-Fluid (VOF) method (16,17) was employed to simulate fluids that are incompressible and immiscible, and the localized slip between fluids was negligible. Surface reconstruction was calculated using an upwind scheme with the use of the Piecewise Linear Interface Construction (PLIC) method (18) and the surface tension was also considered. The simulation was implemented in the transient state. A two-dimensional structured grid was applied to calculate the approximate velocity and time that is consumed for capillary flow filling. In addition, half of a real-size three-dimensional structured grid was generated to simulate real capillary flow and the spread of cells in the channel. For the calculation of capillary force, we set the surface tension (γ) of water at room temperature at 0.0725 N/m, and set the density of water at 1,000 kg/m^3 and the density of air at 1.16 kg/m^3. We also used the spray module on the CFD-ACE software to simulate cells in the channel and we assumed that the diameter of a cell was 8 μm and the density of the cells was 105/mL. To reduce computational time we solved the half domain, and it was acceptable as the channel has a plane of symmetry. Grid-independent simulation solutions were achieved at 47,500 grid cells in the present study. From the simulation results, we finally determined the dimension and shape that satisfied the design requirements.

Microfluidic chip fabrication. Microfuidic chips were fabricated with the microinjection molding process developed by the Nanoentek Corporation (Seoul, Republic of Korea). These chips were made of polymethylmethacrylate (PMMA) and microfluidic techniques were applied, generating natural capillary flow for proper sample loading and even distribution of cells throughout the channels. The microfluidic channel is located in the center of the microfluidic chips, with a width of 75 × 25 mm in size with a height of 0.1 mm, and the actual channel dimensions for sample loading were 51 × 9 × 0.1 mm^3 (Fig. 2C). To achieve regular distribution and fast precipitation of the cells, we employed a backward step structure at one end of the channel. Thus, we could accomplish passive flow velocity control. Figure 2C shows a depiction of the microfluidic chip. The chip was made by the bonding of two plates; one plate was clear and smooth, and the other plate was micro-channeled. The plates were bonded by chemicals that have no accompanying reactions with liquids, cells and any other materials used in biological tests. To generate natural capillary flow, the internal surfaces of the plates were chemically treated to change their wettability with gas plasma. To certify an even distribution of particles in the microchip, we divided the microchip into 43 × 8 partitions with a 1.06 × 0.72 mm area (Fig. 1), each, and used 6-μm fluorescence beads (Invitrogen Corp., San Diego, CA). After the addition of 50 μL of a fluorescence particles solution at a concentration of 1.0×10^6/μL, we counted the fluorescence particles in each partition with a C-Reader (Nanoentek, Seoul, ROK).

Staining of $CD4^+$ T Lymphocytes

For the determination of the number of $CD4^+$ cells in whole blood, a 50-μL whole blood sample was incubated for 15 min at room temperature with 20 μL of CD4-PE (BD Pharmingen, Becton Dickinson Biosciences, Franklin Lakes, NJ) and was diluted 1:20 in 1× phosphate buffered saline (PBS). Then, 50 μL of the diluted sample was loaded onto a plastic microchip for analysis.

Microscopic Cell Counter (C-Reader, Nanoentek)

The disposable plastic chip was mounted on an inverted microscope-type optical system (C-Reader, Nanoentek, Seoul, ROK). The microscopic component of the inverted microscope-type optical system is tabletop size; the size is 262 × 384 × 354 mm^3 and the weight is 17 kg. As a light source, a green laser (50 mW

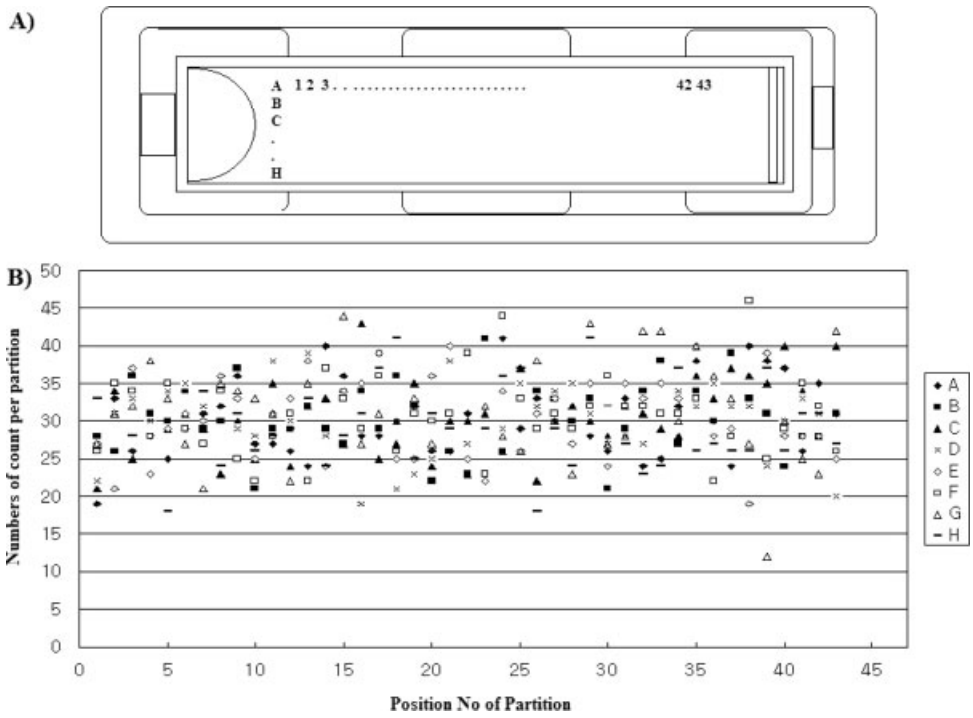

FIG. 1. The particle distribution pattern of microchip. (**A**) The microchip with divided partitions (43 × 8) for evaluation of even particle distribution. (**B**) The result of particle count in each partition.

DPSS laser, HCP, Taipei, Taiwan) at a wavelength of 532 nm was used. Fluorescence was collected by a 5× objective lens and passed through a filter block (600 ± 25 nm) to a charge-coupled device (CCD) camera (1/2 inch sensor, B/W camera, Mintron Enterprise, Taipei, Taiwan) (Fig. 2A). To ensure accurate cell counts, 344 frames of fluorescence images, corresponding to 17.6 μL of a neat sample were processed. Manual focusing was applied and the focusing device was located on the light side of the C-Reader (Nanoenkek, Seoul, ROK). When manual focusing was finished, with a fast stage moving operation, the sample in the chip was scanned. Cell morphology images were acquired with a 20× objective for a real-time morphological assay. The captured image also has a background signal for counting of cells, but manual focusing or the counting program can control and set the optimal level of background signal, with no influence on cell counting.

The C- Reader was interfaced with a PC by a RS232C serial communication cable and a frame grabber board (640 × 480 pixels, 8 bit resolution) obtained from Matrox Electronic Systems (Dorval, Quebec, Canada). It takes 5 minutes for CD4 counting after the sample is inserted in the C-Reader (Nanoentek, Seoul, ROK).

Visual C++ (Microsoft Visual Studio 2005 Standard Edition) based software (Microsoft, Redmond, WA) was used to operate the microscopic cell counter, to count the number of cells and to display the results—a live cell image and a histogram of cell size (Figs. 2B and 2D). As $CD4^+$ lymphocytes with monoclonal CD4-PE (BD Pharmingen, Becton Dickinson Biosciences) are virtually uniform in size, no calibration for varying cell size or morphology is required.

The final concentration of $CD4^+$ T cells in the sample (given as $CD4^+$ T cells/μL) was automatically calculated from the signal of the microscopically captured images.

Reference Standard Methods (Single and Dual Platform Flow Cytometry)

To evaluate the accuracy of the $CD4^+$ counting system, both single and dual platform flow cytometry methods were used for comparison. For the single platform flow cytometry method, the TruCount (BD Biosciences) method was used. For staining, 20 μl of TriTEST CD3-FITC/CD4-PE/CD45-PerCP reagent and 50 μl of whole blood were added to bead-containing TruCount tubes (BD Biosciences) (8). The tubes were incubated for 20 min at room temperature before 450 μl of FACS lysing solution was added. The samples were analyzed on the same day.

For the dual platform flow cytometry method, relative proportions of specific cell subpopulations were obtained using a Cytomics FC500 flow cytometer (Beckman Coulter, Miami, FL), and the absolute number of total leukocytes was determined by use of an automated cell analyzer (Cell-Dyn 4000; Abbott Diagnostics, Santa Clara, CA). Staining was performed with CYTO-STAT triCHROME CD8-FITC/CD4-RD1/CD3-PC5, IgG1-FITC/IgG1-RD1/IgG1-PC5 (Beckman Coulter). The $CD4^+$ T lymphocyte count was calculated from the leukocyte concentration and the percentage of lymphocytes analyzed (8).

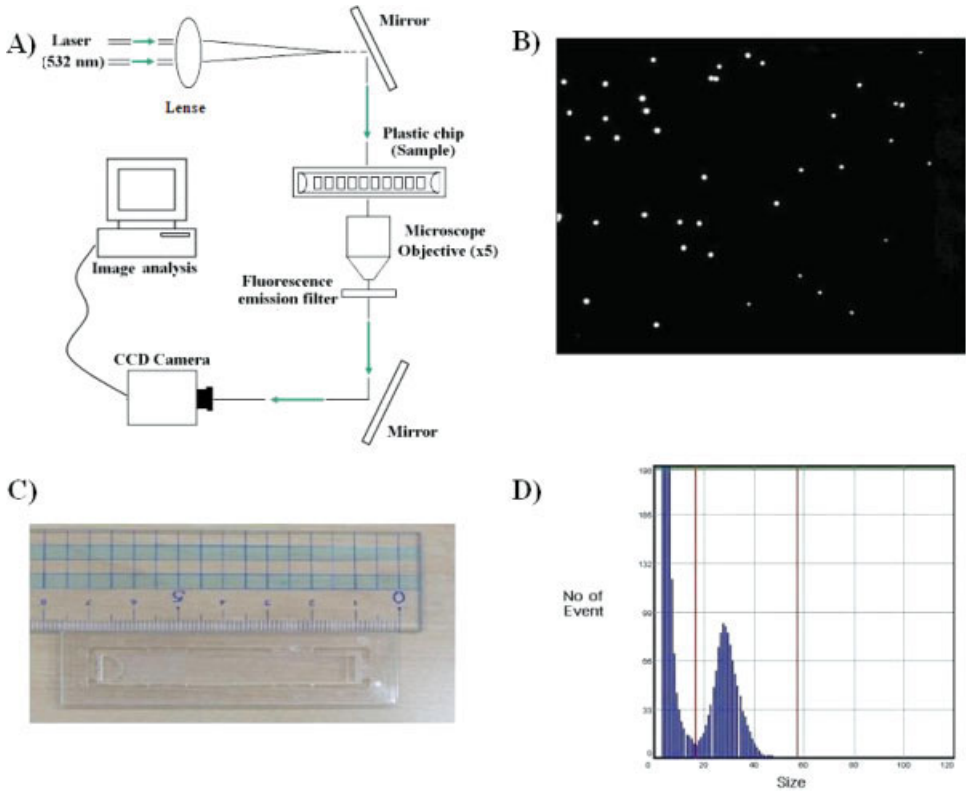

FIG. 2. $CD4^+$ cell counting using a microscopic cell counter. (**A**) Schematic diagram of the $CD4^+$ cell counting system. A stained sample was loaded on the disposable plastic chip and subjected to a green laser light (wavelength of 532 nm), and the fluorescent image obtained by the CCD camera was automatically analyzed with a Visual C++-based software program. (**B**) Image of $CD4^+$ cells obtained in the CCD camera. (**C**) The microchip used for $CD4^+$ T cell counting. **D**) A histogram of cell size after automatic analysis is shown. [Color figure can be viewed in the online issue which is available at www.interscience.wiley.com.]

Clinical Samples

The Korea University Hospital Institutional Review Board approved this study. Blood was obtained from HIV-1-uninfected control participants and HIV-infected participants at Korea University Ansan Hospital between May and October 2007. Informed consent was obtained from a total of 123 individuals, including 35 HIV-infected patients. Among the 88 HIV-seronegative individuals, 45 were healthy individuals, and 43 were cancer patients (e.g., patients with stomach cancer). All of the peripheral blood samples were collected in EDTA-treated tubes and were processed within 24 h after being obtained. Preparation of daily samples for all three methods was performed simultaneously.

Linearity and Reproducibility Study

To avoid intersample variation in the leukocyte concentration, a sample of leukocyte-depleted peripheral blood was blended with a known volume of lymphocytes, separated with Ficoll-Paque (Amersham, Uppsala, Sweden). For the linearity assay, whole blood was diluted 2- to 10-fold using a double filtered erythrocyte concentrate from the same donor. The expected $CD4^+$ lymphocyte concentrations were 500, 200, 100, 80, and 50 cells/μL. To study the precision of the method, samples at each concentration were counted 10 times, and coefficients of variation (CVs) were calculated.

Interference of $CD14^+$ Monocytes with the Absolute $CD4^+$ Counting System

To evaluate the influence of monocytes in this study, 22 monocytosis samples were included. The range of monocytosis was 10–63% (mean, 19.5%). For isolated peripheral blood mononuclear cells, 10 mL of EDTA-treated whole blood was carefully loaded on 3 mL Ficoll-Paque. After washing three times, approximately 4×10^7 cells were resuspended in 320 μL PBS, and the cells were incubated for 20 min at 4°C with 80 μL of CD14-coated micro beads (Miltenyi Biotec, Auburn, CA). The stained cell suspensions were loaded on a LS column (Miltenyi Biotec) and $CD14^-$ cells were collected after elution from the column. The column was washed three times with PBS, and the column was then removed from the separation unit and was transferred to a new collection tube. PBS (5 μL) was passed through the column, and labeled $CD14^+$ cells were collected. A total of 50 μL of the collected cells ($CD14^-$, $CD14^+$) were incubated with 20 μL of CD4 monoclonal antibody for 15 min at room temperature in the dark, and the cells were then analyzed by flow cytometry and the new counting system.

Table 1
Correlation of CD4$^+$ Cell Counts with the use of the Microscopic Cell Counter Method with a Plastic Microchip (MCP) versus CD4$^+$ Cell Counts Obtained Using the Single Platform (1-PF) and Dual Platform (2-pf) Fow Cytometry Methods

	Correlation coefficient (r) and (P-value)[a]			Mean CD4$^+$ count		
CD4$^+$ T counts group	MCP vs. 1-PF	MCP vs. 2-PF	1-PF vs. 2-PF	1-PF	2-PF	MCP
>500 (n = 67)	0.96 (0.00)	0.90 (0.00)	0.95 (0.00)	846.4 ± 243.0	867.0 ± 269.6	866.6 ± 246.6
201–499 (n = 39)	0.84 (0.00)	0.75 (0.00)	0.83 (0.00)	357.8 ± 81.4	357.1 ± 73.6	357.9 ± 74.8
<200 (n = 17)	0.90 (0.00)	0.91 (0.00)	0.93 (0.00)	106.0 ± 47.7	112.0 ± 47.3	121.2 ± 49.5
Monocytosis (n = 22)[b]	0.97 (0.00)	0.95 (0.00)	0.97 (0.00)	436.9 ± 240.5	462.4 ± 264.3	439.4 ± 248.8
Total (n = 123)	0.99 (0.00)	0.98 (0.00)	0.98 (0.00)	589.1 ± 346.8	598.4 ± 362.8	602.3 ± 353.1

[a]A statistical comparison was performed by the use of the Speaman rank order correlation test.
[b]This group is included in each subgroup.

Statistical Analysis

The data was statistically analyzed using SigmaStat 2.0 software (Jandel, Chicago, IL) and Analyse-It software (Analyse-It Software, Leeds, UK). Significance for the relationship between the CD4$^+$ T cell counts obtained by the use of the microscopic cell counter (with the plastic microchip) and by the reference methods was analyzed by use of the Spearman's rank order correlation coefficient method and the paired t test or Wilcoxon signed-rank test,. Significance was considered significant for $P < 0.05$. Linearity was assessed by calculating the Pearson's regression coefficient between the observed and expected values.

Further analysis using Bland-Altman plots was used to assess if there was any systematic bias between the absolute counts derived from each method. The mean differences (bias) between the use of the new method and each reference method were calculated by Bland-Altman plot analysis (19).

RESULTS

We certified the even distribution of the particles in the microchip using 6 μm of fluorescence beads. The

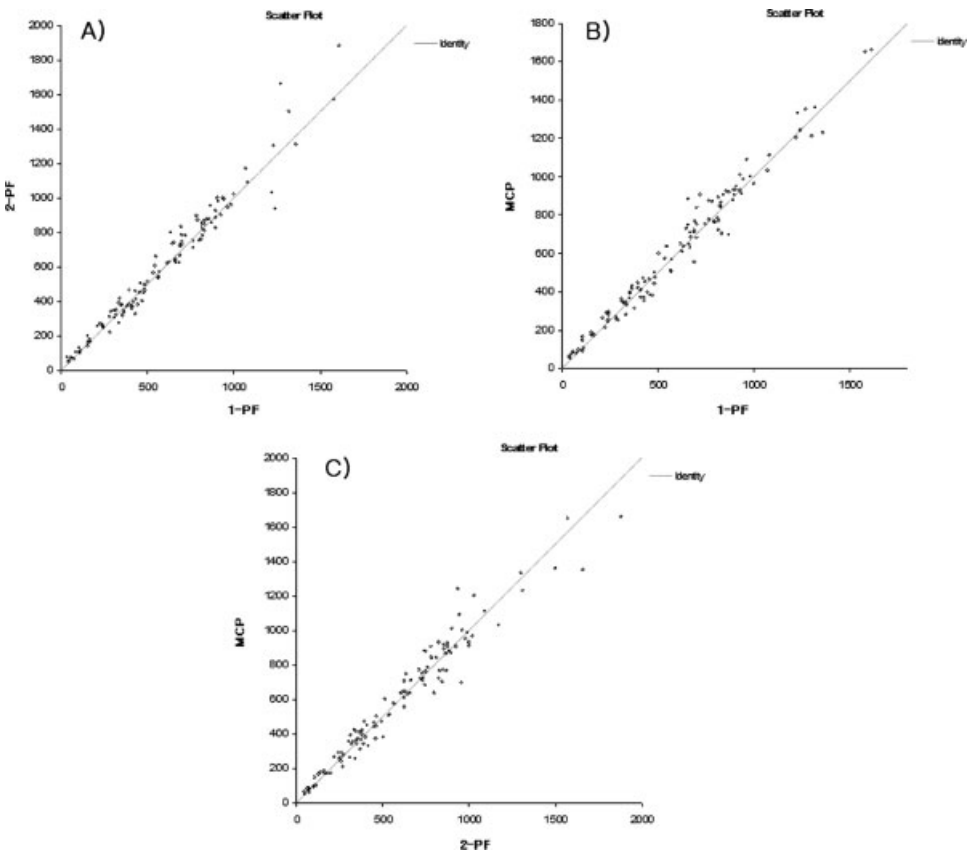

FIG. 3. Comparison of the absolute CD4$^+$ T-lymphocyte counts obtained using flow cytometry or the MCP technique (n = 123). Abbreviations: 1-PF, single platform flow cytometry; 2-PF, dual platform flow cytometry; MCP, microscopic cell counter with a plastic microchip. **A)** 1-PF vs 2-PF; **B)** 1-PF vs MCP; **C)** 2-PF vs MCP.

mean particle number was 30.4 per partition, and the standard variation and coefficient of variation (CV) were 5.3 and 17.4, respectively (Fig. 1).

Samples from participants with a wide range of $CD4^+$ T cell counts were evaluated. The overall correlation coefficients for the absolute $CD4^+$ T cell counts obtained using plastic microchips and the overall correlation coefficients obtained with the use of single or dual platform flow cytometry methods were very high ($r \geq 0.98$) (Table 1, Fig. 3). The difference of the $CD4^+$ T cell counts in clinical samples ($n = 123$) between the dual platform method (598.4 ± 362.8) and the new method (602.3 ± 353.1) were not statistically significant ($P = 0.39$) (Table 2). However, the mean $CD4^+$ T cell counts obtained from the single platform flow cytometry method were lower than the mean $CD4^+$ T cell counts obtained from the use of the dual platform flow cytometry method or from the use of the new method (Tables 1 and 2). The mean difference (bias) between the new method and the dual platform flow cytometry method was 0.4, as determined by Bland-Altman analysis (limits of agreement: −156.6 to 157.4 cells/μL) (Table 2). Slightly higher $CD4^+$ T cell counts were observed with the use of the new method as compared with the use of the single platform flow cytometry method (bias: +13.1, limits of agreement: −101.9 to 128.2 cells/μL; Table 2, Fig. 4).

The new method was sufficiently precise over the expected ranges in artificially diluted samples; the intra-assay mean CV was 6.3%. Five artificially diluted blood samples at $CD4^+$ T cell concentrations of 500, 200, 100, 80, and 50/μL had CVs (repeated 10 times) of 4.3, 3.1, 7.6, 4.1, and 6.7%, respectively, with Pearson's r values of 0.996 (Fig. 5), indicating good linearity.

With respect to discrimination of fluorescence intensity, the CD4 fluorescent intensity of monocytes collected by magnetic cell sorting (MACS) was weak and far less than the fluorescent intensity of the cut-off level ($CD4^+$ T cells) (Fig. 6). There was no statistically significant difference ($P \geq 0.05$) in the $CD4^+$ T cell counts of samples from patients with monocytosis between the use of the new method and either of the flow cytometry methods (Table 2), and an excellent correlation was achieved among all of the methods ($r \geq 0.97$).

DISCUSSION

Of the several methods available for $CD4^+$ lymphocyte counting, flow cytometry is accepted as the standard method. It is, however, rarely available in countries with numerous HIV infected patients, because of the high cost of the equipment and reagents.

In this report, we describe a new $CD4^+$ lymphocyte-counting device that employs a microchip and microscopic cell counter. In a previous report (15), this plastic microchip device was able to measure a minimum concentration of approximately 0.24 WBC/μL. The use of this platform provides an advantage over current conventional devices, as it requires very small volumes (50 μL), and the counting speed is very rapid (5 minutes/

Table 2
Bland-Altman Analysis of the Overall and Stratified Absolute $CD4^+$ Cell Count Obtained Using the Microscopic Cell Counter Method with a Plastic Microchip (MCP) as Compared to the Single Platform (1-PF) and Dual Platform (2-PF) Flow Cytometry Methods

$CD4^+$ T count group	Bland-Altman difference (limits of agreement)			*P*-value[a]		
	MCP-1 PF	MCP-2 PF	1PF–2PF	MCP-1 PF	MCP-2 PF	1-PF vs. 2-PF
>500 (n = 67)	+20.2 (−119.4 to 159.8)	−2.1 (−202.0 to 197.7)	+24.8 (−157.8 to 207.4)	0.02	0.87	0.02
201–499 (n = 39)	+0.1 (−84.2 to 84.5)	+9.5 (−95.9 to 100.5)	−3.2 (−87.8 to 81.5)	0.99	0.78	0.66
<200 (n = 17)	+15.3 (−20.1 to 50.7)	+7.2 (−33.6 to 44.9)	9.7 (−24.6 to 44.0)	0.00	0.28	0.04
Monocytosis (n = 22)[b]	+4.6 (−109.2 to 118.4)	−23 (−162.1 to 112.2)	25.6 (−101.1 to 152.1)	0.08	0.10	0.08
Total (n = 123)	+13.1 (−101.9 to 128.2)	+0.4 (−156.6 to 157.4)	−13.6 (−130.9 to −158.1)	0.00	0.39	0.03

[a]A statistical comparison was performed with the use of the paired-t test or signed rank test.
[b]This group is included in each subgroup.

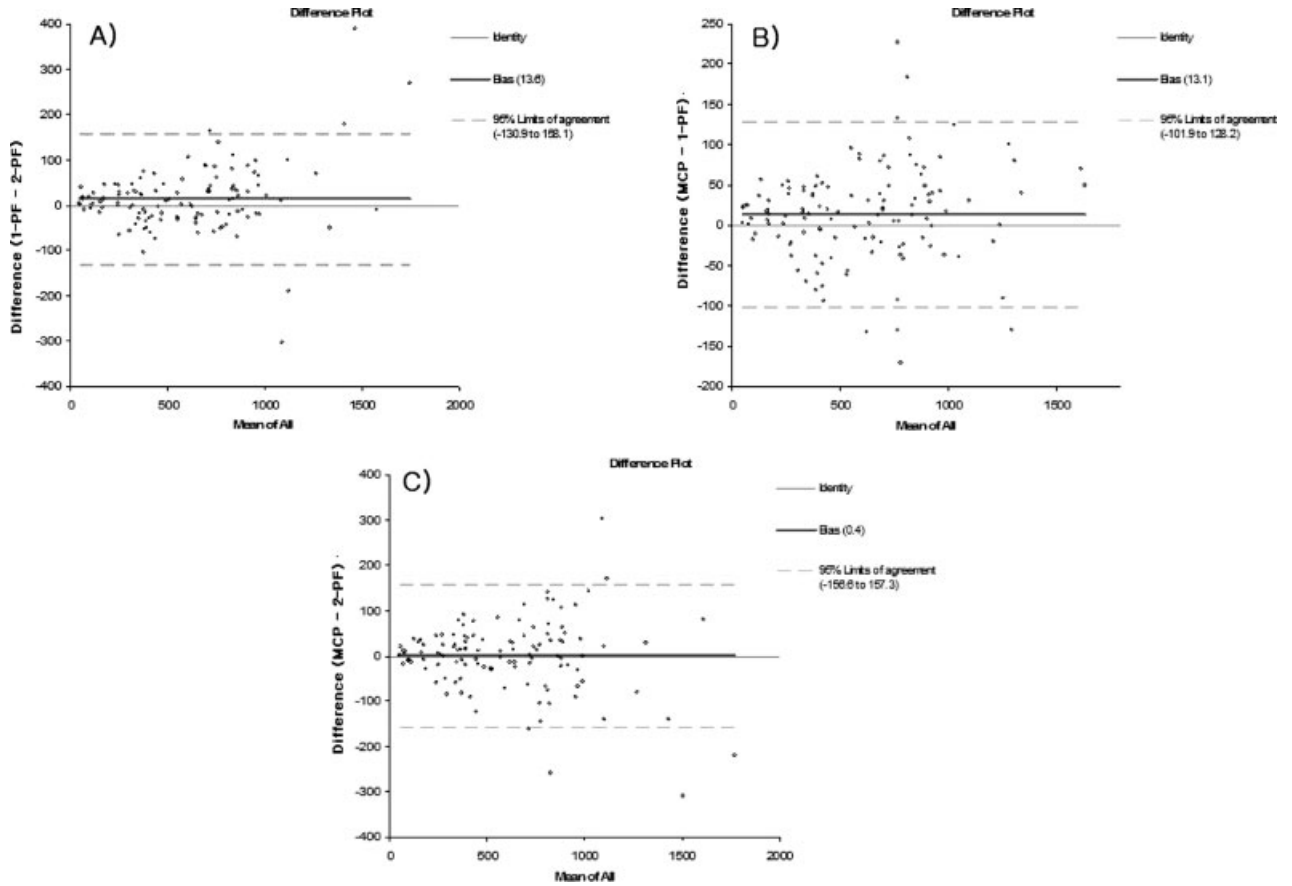

FIG. 4. A Bland-Altman plot to detect differences in the mean $CD4^+$ T lymphocyte counts with the use of 1-PF, 2-PF and the MCP (n = 123). The mean difference (solid line) and the limit of agreement (dashed lines) are indicated. Abbreviations: 1-PF, single platform flow cytometry; 2-PF, dual platform flow cytometry; MCP, microscopic cell counter with a plastic microchip.

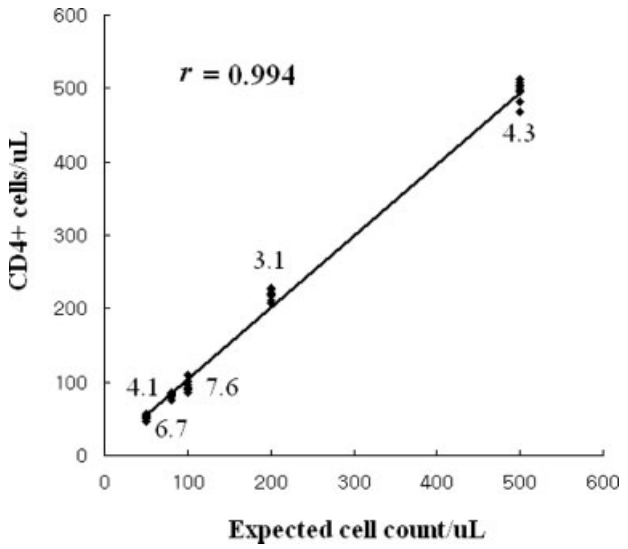

FIG. 5. The correlation between $CD4^+$ lymphocyte counts as determined by the MCP system and the expected $CD4^+$ counts in diluted samples. Whole blood was diluted 2- to 10-fold with a double-filtered erythrocyte concentrate from the same donor. Each concentration of $CD4^+$ lymphocytes was counted 10 times; the linearity was r = 0.996 and the CVs ranged from 4.1 to 7.6.

sample). The $CD4^+$ counts are displayed as cell images and an absolute $CD4^+$ concentration of cells/μL is calculated by use of an image analysis program.

The accuracy and precision of the new $CD4^+$ T cell counting system are essentially equivalent to the accuracy and precision of the dual platform flow cytometry method. Counts were within an allowable level as compared with the findings of other studies (20,21). Emergence of these kinds of systems was expected as an alternative cytometric paradigm (22). Among these new systems, volumetric capillary cytometry is an example (23,24), and the use of imaging instruments for CD4 T cells have been reported (10–14). These systems have adapted similar principles for CD4 counting as compared to our new system.

We believe that the improved accuracy of our method was derived from the control of the sample volume in the microchip chamber and the detection of fluorescence through the translucent chip. Therefore, an important design consideration was the dimensions of the microchip chambers. For biomedical applications involving RBCs, WBCs and cultured cells, it is important to have a simple structure to ensure easy flow. Once a prototype microchannel has been built and the flow of

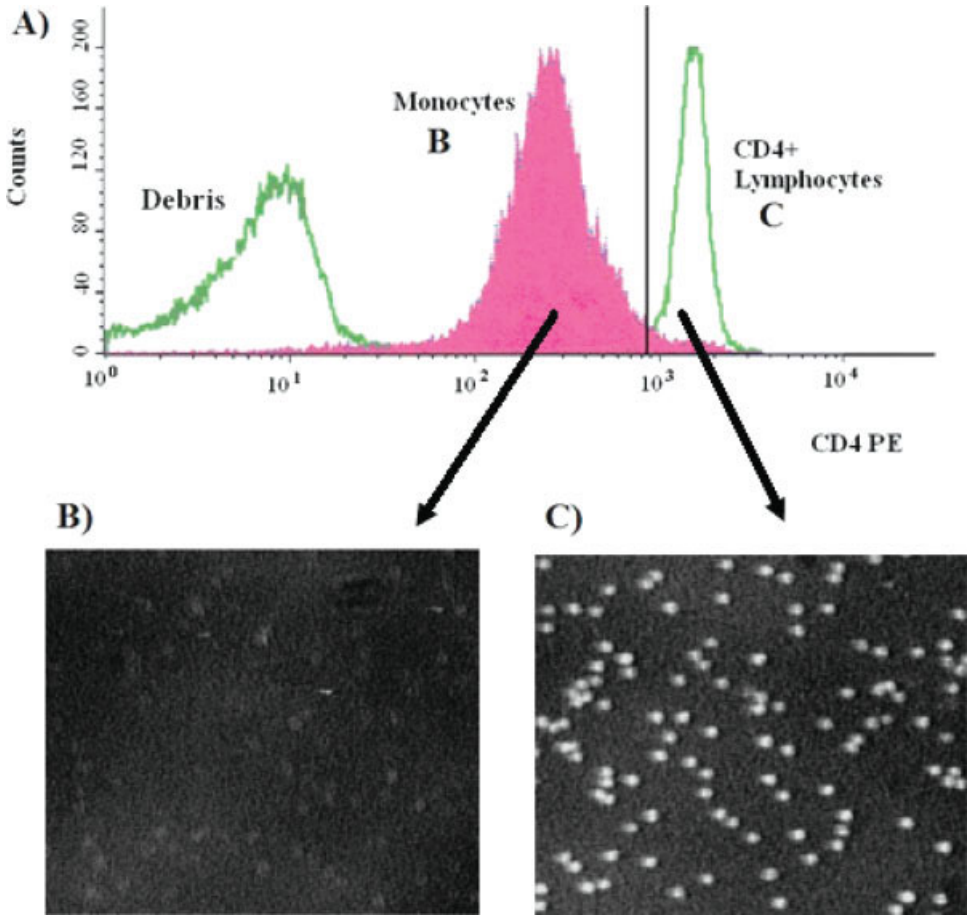

FIG. 6. Discrimination of the fluorescence intensity of the CD4 monoclonal antibody for monocytes and $CD4^+$ T lymphocytes determined using flow cytometry (**A**). A real image of monocytes (**B**) and $CD4^+$ T lymphocytes (**C**) as captured in the MCP system. [Color figure can be viewed in the online issue which is available at www.interscience.wiley.com.]

cells tested, microdevices can be designed so that the units are better suited for specific applications. Compared with flow cytometry, the microchip system can be miniaturized. It has many other advantages, including increased portability, reduced cost, a requirement for reduced amounts of reagents and biological samples required for analysis, and shorter measurement times.

For flow cytometry, the gating strategy is complicated and is not practical for inexperienced users. However, the new system uses a simplified gating strategy that counts only fluorescent cells, in this case only CD4 cells. The population of $CD4^+$ lymphocytes can clearly be discriminated from dim, nonlymphocyte CD4 cells (Fig. 6), and only fluorescent particles are counted in a whole blood sample. In a study of monocytes, we have verified that non-fluorescent particles (debris, erythrocytes, platelets and all non-CD4 leukocytes) do not affect the results. As $CD4^+$ lymphocytes with monoclonal CD4-PE are virtually uniform in size, no calibration for varying cell size or morphology is required for the differentiation of $CD4^+$ T cells from debris. $CD4^+$ monocytes stained with CD4-PE are seen as a very faint dot image and can be eliminated with an instrument setting of intensity cut-off before starting $CD4^+$ counting. However, the addition of a second parameter (e.g., a PE-Cy5 CD3 antibody) would be optimal to eliminate the interference from monocytes for CD4 T cell counts. As monocytes and macrophages also express the $CD4^+$ molecule, they must be identified and excluded from the absolute count. Some studies have used a blocking reagent with CD14 monoclonal antibody-labeled beads added to the CD4 samples to achieve the blocking (6).

This no-lyse, no-wash protocol is practically a one-step procedure that needs only 15 min for incubation of the antibody labeling and 5 min for analysis. Therefore, the $CD4^+$ counting system is suitable for a portable lab-on-a-chip instrument. The new technology is more cost-effective than the use of single and dual platform flow cytometry methods when the cost of the WBC and lymphocyte differential counts, such as the costs of the TruCount tubes and TriTEST are considered.

From our study, we conclude that direct CD4 counting using a microchip with a microscopic cell counter is a precise technology to obtain absolute CD4 counts. The new device is cost-effective and efficient, and permits reliable identification of discrete lymphocyte popula-

tions by different CD4 staining intensities that are adequate with the use of a single monoclonal antibody. This microchip system has many potential applications in diagnostics; thus, it is important to design microchip devices with improved flow resolution and control.

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